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Insecticidal Activity and HPLC Correlation of Thuringiensin from Fermentation and Two-phase Aqueous Separation Processes

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Abstract: Thuringiensin produced by *Bacillus thuringiensis* subsp. *darmstadiensis* was further subjected to a two-phase aqueous separation system. A modified HPLC method and a test for quantitative pathogenicity using the house fly *Musca domestica* were used for analysis of thuringiensin. Within a realistic range of dosages, more effect was observed in the pupal stage than in the larval stage. The percentage effective control rate (ECR) was calculated by (100 − percentage emergence); malformed and non-reproductive adults were considered as emerged. Pupal mortality, pupal weight, and ECR after feeding the three-day-old larvae were the measured response criteria for bioassay. The EC_{50} of thuringiensin for pupae mortality was 1·64 μg ml^{-1} diet, and 0·83 μg ml^{-1} for mortality of adults. Insecticidal activity of the broth increased with fermentation time-course from 9th to 21st hour. The bioassay curve constructed with three-hour sampling interval during the fermentation course had good correlation to thuringiensin content as determined by the HPLC method. In the two-phase aqueous separation system, a maximum of 96·7% ECR was achieved with the bottom salt layer, compared to a value of 46·7% with the upper PEG layer. These results suggest that thuringiensin, prepared through a fermentation and recovery process, is suitable for pest control.

Key words: thuringiensin, *Bacillus thuringiensis* subsp. *darmstadiensis*, fermentation, two-phase aqueous system, insecticidal activity, HPLC

1 INTRODUCTION

The development of alternatives to synthetic chemical pesticides is an ever-accelerating activity around the world today. *Bacillus thuringiensis* Berliner (B.t.)-based insecticides constitute the overwhelming majority of biopesticides which, combined with a variety of emerging opportunities, have created the prospect of the commercialization of a new range of highly effective B.t.-based products.[1] Thuringiensin(β-exotoxin), one of several toxins produced by some strains of *B. thuringiensis*, is a low-molecular-weight, heat-stable nucleotide with insecticidal,[2] miticidal[3] and nematicidal activity.[4] Field-trial results with DiBeta, a thuringiensin-based development candidate from Abbott Laboratories, has demonstrated efficacious control of several species of mites, resistant Colorado potato beetles, and lygus bugs. Scaled-up runs of the carbon-limited fermentation showed very consistent and reproducible process translation from the 55-litre scale to the 24 000-litre scale and even up to the 80 000-litre scale.[5]

Thuringiensin has a wide range of activity against members of several insect orders. However, because of conflicting results in bioassays of the toxins produced by several strains of B.t., it has been reported that more than one heat-stable exotoxin is produced, such as sigma-exotoxin.[6] An important factor for industrial

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scale-up of thuringiensin production is the quantification of toxin concentration in relation to bacterial growth. The main limitation in studying these factors is the inability to quantify thuringiensin concentration in fermentation broth. Several laboratories have reported the use of high-performance liquid chromatography (HPLC) for the detection and quantification of thuringiensin[7–9] as an alternative to the tedious and time-consuming bioassay. Bekheit *et al.*[10] developed an ELISA for thuringiensin, which opened new prospects for analytical techniques. Wu *et al.*[11] and Tzeng *et al.*[12] also found variations of thuringiensin content during fermentation time-course and in preparations using a two-phase aqueous separation system monitored by HPLC. In fact, the insecticidal activity should be evaluated by a bioassay-correlated HPLC quantification method.

In the present study, the insecticidal activity of thuringiensin on house fly (*Musca domestica* L.) during fermentation and further recovery process was investigated, and the following points were considered: (1) correlation between bioassay and HPLC analysis, (2) the role of thuringiensin in the heat-stable exotoxins and (3) the synergistic effect between thuringiensin and endotoxins of *B. thuringiensis*. Our results clearly demonstrated that the production of thuringiensin based on B.t. depends on the combination of fermentation and recovery processes, and can be monitored by instrumental analysis and biological assays.

2 MATERIALS AND METHODS

2.1 Micro-organism and batch fermentation

The culture of *B. thuringiensis* subsp. *darmstadiensis* (HD199) isolate was obtained from Dr H. de Barjac, Pasteur Institute, Paris. The organism was maintained on agar slants of Schaeffer's sporulation assay medium[13] at 28°C and subcultured at monthly intervals. For batch culture, a SMB medium of the following composition was used: soy protein, 30·0; molasses, 20·0; potassium dihydrogen phosphate, 5·0; dipotassium hydrogen phosphate, 5·0; $Na(NH_4)PO_4.4H_2O$, 1·5; magnesium sulfate, 0·05; manganese sulfate, 0·03; ferrous sulfate, 0·01; calcium chloride, 0·05 g litre^{-1}. All media ingredients were autoclaved together, with the exception of magnesium sulfate, manganese sulfate, ferrous sulfate and calcium chloride, which were added aseptically using concentrated sterile solution. The pH of the medium was adjusted to 7·0 with aqueous sodium hydroxide (4M) and 85% phosphoric acid + water (1 + 20 by volume). The seed culture was prepared by inoculating a loopful of cells from a nutrient agar (NA) stock plate culture into 50 ml of SMB medium and grown in a shaking incubator at 30°C for 24 h. After growth, the cells were transferred into a 3-litre fermentor, where temperature and pH were controlled at 30°C and 7·0, respectively. The conditions of aeration and agitation were 0·5 VVM and 500 rev min^{-1}, respectively.

2.2 Phase separation in polymer–salt system

A two-phase system consisting of PEG (150 g litre^{-1}) and dipotassium phosphate (100 g litre^{-1}) in the supernatant of fermentation broth was prepared. The system was shaken for 60 min in a wrist shaker (Lab-line Instruments Inc., Melrose Park, Ill 60160, USA). The solution was then centrifuged for 10 min at 4960 *g*, and allowed to settle in a separation funnel, which was then kept at room temperature overnight to separate the two phases. Samples of the upper layer were removed with a pipette. The bottom layer was sampled using a pipette with positive pressure, to avoid contamination. Thuringiensin concentrations in the two phases were subject to bioassay after series of dilutions. In addition, polyethylene imine (PEI) was used to facilitate clarification and precipitation. The thuringiensin-containing supernatant was applied to PEI solutions of concentration ranging from 0·5 to 15 ml litre^{-1} at equal volume through shaking by wrist shaker for 1 h. The mixture was then centrifuged at 8820 *g* for 20 min. The resulting precipitate was resuspended with 5 ml deionized water and shaken again by wrist shaker for 1 h. The suspensions were further centrifuged at 8820 *g* for 20 min. The final supernatant was subject to HPLC analysis and bioassay.

2.3 Standard and HPLC analysis

Thuringiensin (β-exotoxin) standard was obtained from the Pasteur Institute, Paris, by the courtesy of Dr H. de Barjac. HPLC assays were performed isocratically on a Waters P-Bondpak C18 column (30 cm by 3·9 mm ID). Mobile phase of KH_2PO_4 (50 mM; pH 2·8) was used at a flow rate of 2·0 ml min^{-1}. The mobile phase was filtered through a 0·45-μm polycarbonate membrane. Solid samples and standards were dissolved in deionized water. Solid samples that did not readily dissolve in deionized water were acidified to pH 2·8 with phosphoric acid. Liquid samples and dissolved samples were diluted to a concentration of approximately 0·1 mg ml^{-1} thuringiensin and filtered through 0·45-μm disposable nylon 66 syringe filters. Samples were then injected into the HPLC. Instrumentation included an M6000A pump, a 501 autosampler equipped with a 20-μl sample loop, a SF 770 variable-wave-length UV detector set at 260 nm, and a Chromatopac-R6A integrator as described by Campbell *et al.*[8]

2.4 Bioassay using house fly *Musca domestica*

Three-day-old house fly larvae obtained from laboratory colonies reared at 30°C and 50% RH were used in the bioassay. One millilitre of supernatant or test solution was thoroughly mixed in a plastic cup with 15 g of artificial diet (according to Peterson[14]). Ten three-day-old house fly larvae were introduced into the cup. Three replications were maintained. The larvae were incubated for 10 days (checked in the fifth day post-inoculation for weight of live and dead pupae) at 30°C and 50% RH. The number of dead and malformed pupae were recorded at the end of the incubation period. The Duncan's MRT and Probit analysis procedure of the SAS were used to determine the effective control rate (ECR). The percentage ECR was calculated by (100 − percentage emergence); malformed and non-reproductive adults were included in emergence. Time-course analyses of fermentation broth was carried out at two doses (1×, 10×), compared to serial doses HPLC analyses of thuringiensin standards.

3 RESULTS AND DISCUSSION

3.1 Quantitative pathogenicity of thuringiensin

Toxic effects attributed to thuringiensin should be treated with caution, since only a few studies have used pure or partially purified thuringiensin. Most results were obtained with autoclaved supernatants of *B. thuringiensis* cultures or with commercial preparations containing spent culture medium, including endotoxin crystals and spores. In our study, thuringiensin in the supernatants (from *B. thuringiensis* subsp. *darmstadiensis* fermentation broth) was quantified by HPLC and tested by house fly bioassay. Bioassay response criteria such as pupal weight, pupal mortality and effective control rate (ECR) were chosen. Data from bioassays of both pupae and adults produced a positive dosage–mortality response curve. The EC_{50} for pupae was 1·64 $\mu g\ ml^{-1}$ diet, and for adults, 0·83 $\mu g\ ml^{-1}$ diet. Pupal weight decreased rapidly at thuringiensin concentration higher than 12·5 $\mu g\ ml^{-1}$ diet, the decrease in pupal weight perhaps being due to malformation, and/or the inhibition of growth by the toxin. The higher pupal mortality and ECR were combined with a reduced pupal weight as shown in Fig. 1. Both malformation and inhibition of growth by toxin will lead to decrease of pupal weight. As thuringiensin concentration increased, pupal mortality and ECR increased. A linear regression model best described the relationship between concentration and mortality estimate: for pupae, $Y = 1{\cdot}14 + 2{\cdot}78\ X$ ($r = 0{\cdot}99$); and for adults, $Y = 1{\cdot}98 + 2{\cdot}76\ X$ ($r = 0{\cdot}99$). It is obvious from these trials that a low chronic concentration of thuringiensin

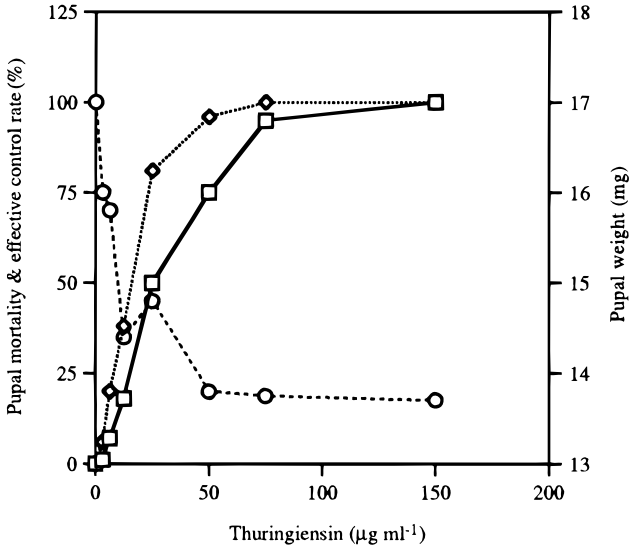

Fig. 1. (—□—) Pupal mortality (—◇—) ECR and (- - ○ - -) pupal weight at different thuringiensin concentrations.

significantly lowered emergence rate compared with the control.

No accurate estimate of LC_{50} was available for larvae because of their high tolerance to thuringiensin, which contradicts the earlier work of Bond *et al.*[15] Hoy and Ouyang[16] indicated that treated larvae were unable to develop into adults of *Tetranychus pacifius* (Mcg.) and *Metaseiulus occidentalis* Nesb. However, the influence of thuringiensin on development rate is difficult to interpret, because mortality and development rate are not independent. It is difficult to make direct comparisons between these data and those of other investigators, because of differences in species, treated foods (leaf or artificial diet), incubation period and incubation condition of bioassay. However, from a practical point of view, these results suggest that the economic benefits of application might be rather high, because the amount of thuringiensin (applied in the form described in this paper) required to produced 50% ECR is very low. In this research, those larvae exposed to thuringiensin through diet during the third-day-old stage remained in the fifth stage (larvae) longer than control, which prevented pupation and subsequent emergence into adults. Similar observations can be found in the above cited literature. Development time in the fifth stage for larvae that survived from intoxication was *c.* 2–3 days longer than that for untreated larvae. During the prolonged development, intoxicated larvae apparently become inactive.

In investigations by other researchers, thuringiensin affected most larvae, probably because large amounts of supernatant were used. When supernatants of lower thuringiensin concentration were used, larvae were still able to develop to pupae, but many of these were malformed. Malformed pupae were defined by comparing with normal ones in terms of shape. Even when pupation was apparently normal, most did not emerge,

and failed to expand their wings, as shown in Plate 1. This result is in agreement with those reported by Briggs[17] and Cantwell *et al.*[18] Recently, researchers have indicated that homometabolous arthropods are susceptible to thuringiensin, which produced teratogenicity;[19] even missing tarsal segments in spider mite have been observed.[3] The action of thuringiensin contributes differently to the quantitative pathogenicity at different life stages. High growth rates and physiological processes such as metamorphosis that occur in immature insects require higher rates of RNA synthesis than are necessary for the relatively slower growth of adults.[20] Therefore, greater sensitivity to the toxic effects of thuringiensin would be expected during the immature stages, which, in turn, will affect the development of the pupal stage and even adults. Mohd-Saleh and Lewis[21] reported that applying high concentrations of thuringiensin to neonate fall armyworm, European corn borer and black cutworm larvae caused less mortality after seven days of exposure than did lower concentrations. They attributed this phenomenon to the possibility of feeding deterrence. In our observations, increased dosages resulted in increased mortalities.

3.2 Bioassay and HPLC analysis of thuringiensin for fermentation process

HPLC has been used by several investigators to detect and quantify thuringiensin produced as an excreted metabolite from various subspecies of *B. thuringiensis*. The assay was rapid and quantitative for purified thuringiensin standards. However, peak height failed to correlate thuringiensin concentration in crude culture filtrate with biological activity toward house fly larvae. Johnson and Peterson[22] commented that HPLC had only limited application in toxin detection and quantification. On the other hand, many publications have reported that dilutions of fermentation broth caused mortality of insect larvae but did not provide any information on thuringiensin concentration which fluctuates during the process of fermentation. In this study, thuringiensin formation during the vegetative phase and sporulation was measured (Figs 2 and 3). The dissolved oxygen (DO) of the medium fell to a minimum as cell number increased, at 15 h fermentation, and then increased sharply with increasing spore numbers until 24 h (Fig. 2). It was observed that there was a good correlation among exponential growth, DO of the medium, and thuringiensin yield. Moreover, when DO was depleted and cell growth ceased there was a continuous increase of thuringiensin concentration. Sporulation commenced 3–4 h after DO depletion. Maximum thuringiensin production occurred at the early log phase of sporulation as shown in Fig. 2. The use of spore-count data to select appropriate media for thuringiensin production could be justified by the correlations demonstrated between spore numbers and thuringiensin formation. Thuringiensin yield of 1·34 mg ml^{-1} obtained in 3-litre fermentation com-

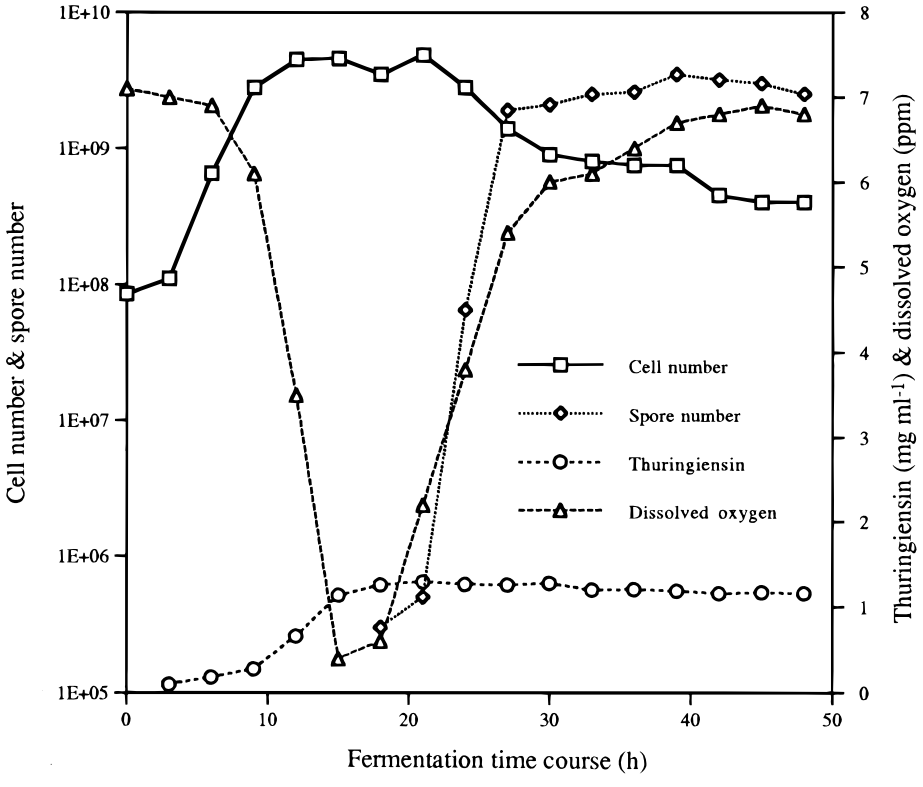

Fig. 2. Dynamics of bacterial growth, spore formation, dissolved oxygen and thuringiensin biosynthesis on *Bacillus thuringiensis* in 3-litre fermentation.

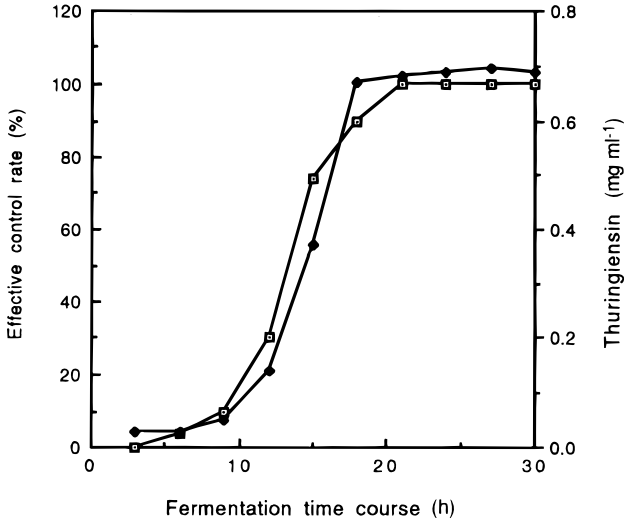

Fig. 3. Monitoring of thuringiensin of *Bacillus thuringiensis* throughout fermentation by (◆) HPLC and (⊡) bioassay with the house fly, *Musca domestica.*

pared favourably with other quoted values. Abrosimova *et al.*[23] obtained maximal yields of 0·730 μg ml^{-1} by optimizing the amount of mineral salts added in the medium.

In the batch fermentation of thuringiensin from *B. thuringiensis* using SMB medium (Fig. 2), the spore concentration did not reach maximum until 24 h after inoculation, whereas sporulation began at 18 h (time of lowest DO). Figure 3 compares the results of bioassay and HPLC analysis of thuringiensin in the supernatant along the course of fermentation. The level of thuringiensin in the medium rapidly increased during the exponential growth phase (10–16 h), and reached a maximum of 0·73 mg ml^{-1} which was maintained after 17 h. Figure 3 reflects a strong correlation between the HPLC analysis of thuringiensin in the supernatant and bioassay (expressed in ECR). Figure 4 shows a typical HPLC chromatogram of the supernatant of the fermentation broth and that of the thurigiensin standard.

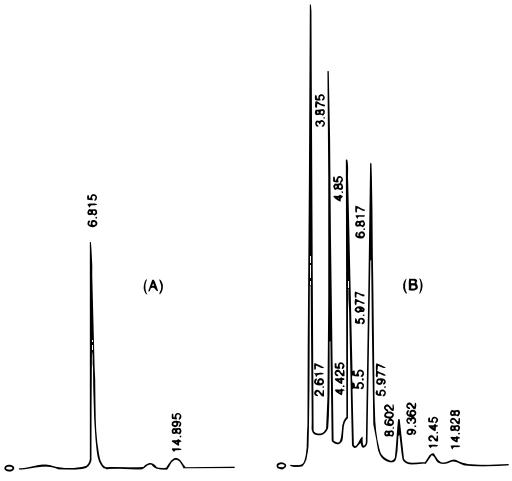

Fig. 4. HPLC chromatogram of (A) thuringiensin standard, (B) supernatant of fermentation broth. The peak at 6·8 min was the thuringiensin.

3.3 Effect of downstream processing on insecticidal activity

Separation processes in biotechnology are defined by the nature of the product and its applications. High degrees of purity which approach molecular homogeneity may be required for some products, whereas simply the absence of inhibiting factors is acceptable for others. The optimal conditions for large-scale recovery of thuringiensin from broth are needed. Aqueous two-phase systems offer great potential for the continuous extraction and purification of target biomolecules from a mixture with low concentration of desirable ingredients, for industrial application.[24–26] Bioassay results obtained with the different preparative process of fermentation broth are given in Table 1. Broth containing thuringiensin, endotoxin crystals, spore cell debris, and residual medium, from fermentation without centrifugation gave higher insecticidal activity than the supernatant. This result might be ascribed to synergism between thuringiensin and crystal proteins, which made the mixture more toxic than expected. Synergism occurring in combinations of *B. thuringiensis* and thuringiensin was reported by Muller and Harper,[27] but the target insect was not house fly. Tabashnik[28] suggested that lack of an appropriate test for synergism could result in misleading conclusions. If synergism occurs, the toxicity of a mixture cannot be estimated from the effectiveness of the individual ingredients.

Practically speaking, the downstream separation process is as important as fermentation *per se.* The bioassay described above can be used to evaluate the validity and efficiency of downstream separation process. A polymer–salt system with 150 g litre^{-1} PEG 8000 and 100 g litre^{-1} K_2HPO_4 was used to separate thuringiensin from supernatants in a cell-free crude extract. As shown in Table 1, insecticidal activity (pupal mortality) in the salt phase with thuringiensin was about twice

TABLE 1
Insecticidal Activity of Thuringiensin against the House Fly, *Musca domestica* through Downstream Preparation of the Supernatant from *Bacillus thuringiensis* Fermentation Broth at 20 × Dilution

Downstream sample preparation	*Pupal mortality* (%)	*Effective control rate* (%)
Fermentation broth	90·00	100·00
Supernatant from broth	66·70	86·70
Supernatant after autoclaving	73·70	83·30
PEG layer in two-phase system	33·30	46·70
Salt layer in two-phase system	66·70	96·70
Supernatant for PEI precipitation	72·40	96·60

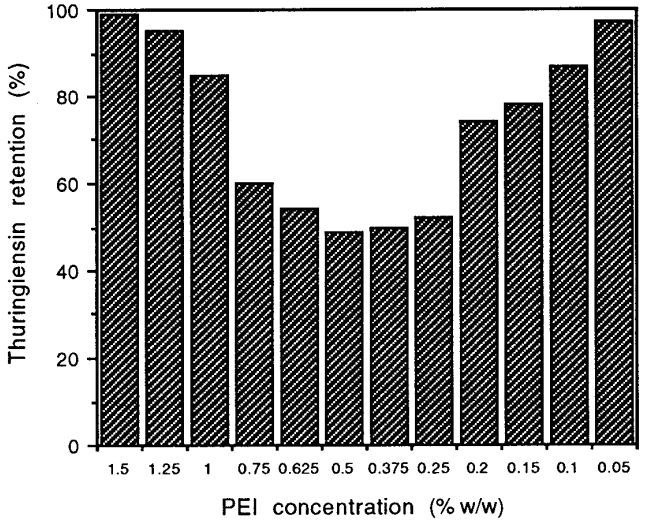

Fig. 5. Retention of thuringiensin in the supernatant of *Bacillus thuringiensis* fermentation broth by polyethylene imine (PEI) precipitation of serial concentrations.

that in the PEG phase at 20 × dilutions of the supernatant, where thuringiensin distribution factor was 0·1651 mg/0·8644 mg = 0·19 (determined by HPLC). Table 1 indicates that PEG and K_2HPO_4 used in the thuringiensin recovery process did not affect final insecticidal activity. For salting-out undesirable components, the highest thuringiensin retention in the supernatant in a series of PEI concentration tests was obtained on 15 g kg^{-1} PEI as given in Fig. 5. Nevertheless, the efficiencies of precipitation of both the higher and lower level PEI in preparations were similar, therefore, the 0·5 g kg^{-1} PEI treatment was chosen for bioassays in this research. The use of 0·5 g kg^{-1} PEI was a relatively simple technique to retain thuringiensin, but with high ECR of 96·6% at 20 × dilution.

In conclusion, liquid chromatographic and bioassay data clearly demonstrated that thuringiensin is the only major exotoxin existing in the supernatant of *B. thuringiensis* fermentation broth. The experiments showed that insecticidal activity in fly bioassay at a three-hour sampling interval from submerged cultivation is strongly related to thuringiensin quantity as shown by HPLC analysis. The possibility of instrumental on-line monitoring (of thuringiensin concentration) of fermentation should be emphasized. The insecticidal activity of the supernatant from broth depends not only on the course of submerged growth, but also on the recovery process.

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